Action of Heat on Cow κ-Casein. Heat Caseino-Glycopeptide

Abstract

The main casein components were somewhat degraded when sterilized. The hydrolytic action of heat is not the same as that of rennin. However, for κ-casein, the actions of rennin and of heat (120 C—20 min) seem to be similar. A heat caseino-glycopeptide was isolated and it had nearly the same amino acid composition as the rennin caseino-glycopeptide. These data again suggest the existence of a very labile linkage in κ-casein which was easily split by different procedures.

Heat degradation of caseins is of great interest to explain phenomena which occur during milk processing. It could also give information on the chemical structures of these proteins, comparable to chemical and enzymatic hydrolysis. Dephosphorylation of whole casein, accompanied by a liberation of nonprotein substances (NPN), was demonstrated long ago by Howat and Wright (7) under severe conditions of heating (120 C, 5 hr). Less liberation of NPN was also observed during heating of milk for shorter times (13, 15). More recently the degradation of whole casein and of Warner's Fractions β and α (this latter contains α_s and κ-caseins) was investigated by Belec and Jenness (4). Phosphorus appeared in inorganic form after heating and β-casein was less affected than α-casein insofar as phosphorus and nitrogen liberation was concerned. Similar results were obtained for phosphorus by Swirsky et al. (16).

We reported some preliminary investigations on the degradation of milk proteins by different heating processes, closely related to sterilization (2). After heating whole casein at 120 C for 20 min, we found residual caseins of low-solubility, non-dialyzable peptides containing phosphorus and sialic acid, and low molecular weight peptides, eliminated by dialysis against water. We concluded that most of the NPN is in the large peptides and that organic phosphorus is liberated first. Probably the same is true for sialic acid, according to de Koning et al. (6).

Our objectives were concerned with the degradation of cow κ-casein. We evaluated the extent of hydrolysis at 120 C, by comparison with other casein fractions. Then we attempted to isolate and to purify the large peptides liberated during heating of cow κ-casein. Finally, we analyzed a peptide similar to the caseino-glycopeptide liberated by rennin action.

Experimental Procedures

Preparation of caseins. Whole casein was prepared from pooled skimmilk by precipating with HCl at pH 4.7, washing the casein twice with water, dissolving it again by adding NaOH at pH .7, precipitating once more at pH 4.7, and washing with water, ethanol, and ethyl ether.

κ-Casein was prepared by the method described by McKenzie and Wake (14). β-casein according to Achaffenburg (3), and α_s-casein according to Thompson and Kiddy (17).

Heating of proteins. One or two per cent solutions of the proteins were adjusted to pH 6.7; 5 ml were placed in thin-walled sealed glass tubes, 6 cm in diameter, and heated in a bath of mineral oil. Heating time was recorded from the time the required temperature was reached in a control tube containing a thermometer. The tubes were shaken during heating.

The NPN was determined after precipitating proteins either at pH 4.6 or by 12% trichloroacetic acid (TCA).

Electrophoretic examinations of the soluble reaction products were made on Whatman no. 1 paper (20 v/cm; 2 hr; volatile buffers containing pyridine and acetic acid, pH 6.5). The revelation was made either with ninhydrin or with the Schiff reagent after oxidation with periodic acid (12), to characterize sugars. The electrophoretic methods employing gels gave poor results, as the peptides probably diffused in the fixing and staining baths.

Electrophoretic examinations of heated casein solutions were made on cellulose acetate strips (20 v/cm; 2 hr; phosphate–citric acid buffer containing 6 M urea, pH 7.1) or on starch gel (15 v/cm; 18 hr; 4 C; trishydroxymethylaminomethane–citric acid buffer containing 7 M urea, pH 8.6).

[1] Seventeenth communication on caseins.

TABLE 1

Nonprotein nitrogen released by heating of casein at 120 C during various times (NPN % of protein nitrogen)

2% Casein solutions	Before heating	Increase after heating for			
		10	20	40	80
		(min)			
1) TCA-NPN					
Whole casein	0.45	0.37	1.15	1.72	2.25
κ-Casein	1.10	—	1.34	1.87	2.72
α_sCasein	0.38	0.43	1.08	1.34	2.04
β-Casein	0.44	0.20	1.01	1.48	1.93
2) pH 4.6—NPN					
Whole casein	0.75		2.9		4.7
κ-Casein	4.80		3.0		5.1
α_s-Casein	0.75		4.1		6.9
β-Casein	0.54		2.4		3.5

Results

Release of nonprotein nitrogen at 120 C. Table 1 shows the results of experiments concerning the influence of the heating time at 120 C on the amount of NPN. The production of NPN substances is higher at 80 than at 20 min. However, for our work, we decided to use a temperature of 120 C for 20 min, because it is similar to sterilization. We emphasize that the most soluble substances at pH 4.6 were obtained with α_s-casein and after treatment by 12% TCA with κ-casein. The least amounts were always obtained with β-casein.

Modification of the isoelectric pattern of heated caseins (120 C for 20 min). Zone or gel electrophoreses in urea were made with whole casein and casein fractions. The patterns after heating were usually not as regular as before. The bands corresponding to the main casein fractions were not clearly defined and a supplementary band near the starting point was observed and sometimes it was slightly displaced towards the cathode, in the position for the paracasein resulting from the action of rennin on κ-casein (Fig. 1). With heated pure κ-casein, some insoluble material remained at the starting point, with buffers having a pH of 7.0 or 8.6, but not with a buffer of pH 9.2 (acrylamide gel). β-Casein also gave a new slow moving band after heating, not observed with α_s-casein; however, the bands corresponding to α_s-casein were always weakened. The low molecular weight substances eventually obtained during heating were probably eliminated by staining.

The heat caseino-glycopeptide soluble in 12% TCA obtained from κ-casein. The first experiments we did to purify the soluble substances released by heating were based on our previous work on the caseino-glycopeptide obtained after rennin digestion (1). The heated κ-casein solu-

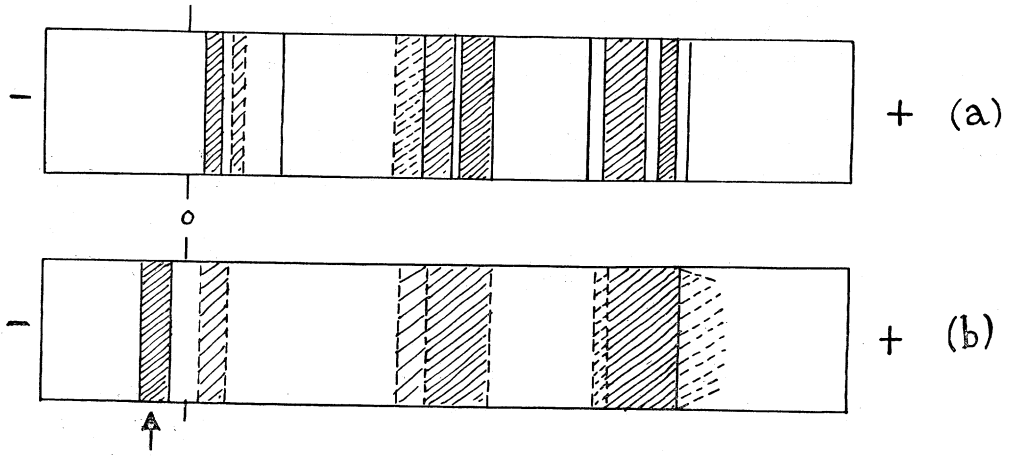

FIG. 1. Electrophoresis of whole casein on cellulose acetate strips (pH 7.0; 6 M urea, 20 v/cm, 2 hr)
a) Before heating. b) After heating (120 C—20 min).

tion was treated with an equal volume of 24% TCA and the supernatant separated by centrifugation, extracted by ethyl ether, dialyzed against water, and freeze-dried.

Two preparations were made: a) was heated in an autoclave at 120 C for 20 min, and b) was heated for 20 min in an oil bath at 120 C, with constant shaking of the tubes. Because of the slow release of pressure, the holding time for a) was longer than for b).

Amino acid compositions of the two heat caseino-glycopeptides were determined. The amino acid residues on the basis of one residue of leucine per mole were calculated (Table 2), and compared with rennin caseino-glycopeptide (molecular weight 8,000) (8). There is much

TABLE 2

Amino acid composition of heat caseino-glycopeptide from κ-casein
(Residues/mole calculated on the basis of one leucine)

	Heat caseino-glycopeptide soluble in 12% TCA		Heat caseino-glycopeptide prepared by chromatography[a]	CGP[b]
	a)	b)		
Aspartic Acid	4	5	4	4
Threonine	7	9	10	9
Serine	6	6	4	6
Glutamic Acid	10	14	10	9
Proline	7	5	6	7
Glycine	4	1	1	1
Alanine	3	4	4–5	5
Valine	5	5	5	5
Cystine (half)	—	—	—	—
Methionine	—	1	1	1
Isoleucine	3	4	5	5
Leucine	1	1	1	1
Tyrosine	—	tr	—	—
Phenylalanine	tr	1	—	—
Lysine	5	3	3	3
Histidine	—	tr	—	—
Arginine	1	tr	tr	—
Total	56–57	59	54–55	55

[a] Peak D, Fig. 3.
[b] Rennin caseino-glycopeptide (8).

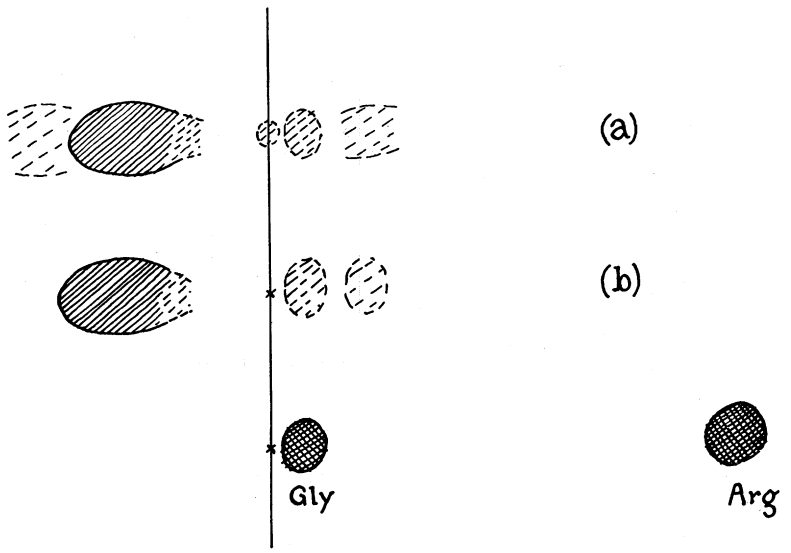

FIG. 2. Paper electrophoresis of heat caseino-glycopeptide a) and rennin caseino-glycopeptide b). (Whatman no. 1; 20 v/cm; pH 6.5; 2 hr.)

similarity between these three substances. The molar ratios of the heat caseino-glycopeptide b) are very similar to those of the rennin caseino-glycopeptide and identical for six amino acids (Thr, Ser, Gly, Val, Leu, Lys).

Also, the electrophoretic pattern at pH 6.5 of the heat caseino-glycopeptide b) was similar to the rennin caseino-glycopeptide, as most of the ninhydrin positive material migrated toward the anode (5) (Fig. 2).

Tentative fractionation of heated κ-casein solutions by gel filtration and electrophoresis. In a previous publication (2) we were unable to obtain a fair separation of the peptides from heated whole casein solutions on dextran or polyacrylamide gels. We again applied this procedure to the heated κ-casein solutions by using various Sephadex columns (from G 15 to G 200) with pyridine–acetic acid buffer (pH 6 and 7), phosphate buffer (pH 7), 3 to 15% butyramide solution, and 3 to 20% dimethylformamide solution. We did not obtain a satisfactory fractionation during these essays. Some peptide material was obtained before complete elution of the protein, but even rechromatography did not produce a complete separation.

We finally tried preparative paper electrophoresis by preparing a small amount of the negatively charged component, which had the same mobility as the rennin caseino-glycopeptide. This peptide was free from Cys, Tyr, and Phe, like the rennin caseino-glycopeptide, but there were large differences in certain amino acid ratios (Ileu/Leu, Thr/Ser).

Chromatography on DEAE- or CM-cellulose did not improve fractionation of the heated κ-casein solutions.

Chromatographic separation of the soluble substances obtained from heated κ-casein after a preliminary deproteinization. Isolation of a heat caseino-glycopeptide. Although an acidic deproteinization was avoided during preliminary experiments for fear of chemical degradation, we finally tried to remove the protein material by lowering the pH to 4.6 before chromatography. Comparative experiments were made with nonheated casein, to try to evaluate the effects of the heating.

All the substances soluble at pH 4.6 were applied on a DEAE-cellulose column and the fractionation achieved by a stepwise elution with 0.5 M pyridine–acetic acid buffers at different pH values. Figs. 3 and 4 show the elution diagrams and the electrophoretic patterns of the different peaks (on paper at pH 6.5). The eluted substances were detected with ninhydrin after alkaline hydrolysis. The proportions estimated were in agreement with those calculated from the dry weights, but this was not the case when the proportions were evaluated by absorption at 280 mμ. The total amount of the soluble substances in the control experiment represented only 25% of that obtained from

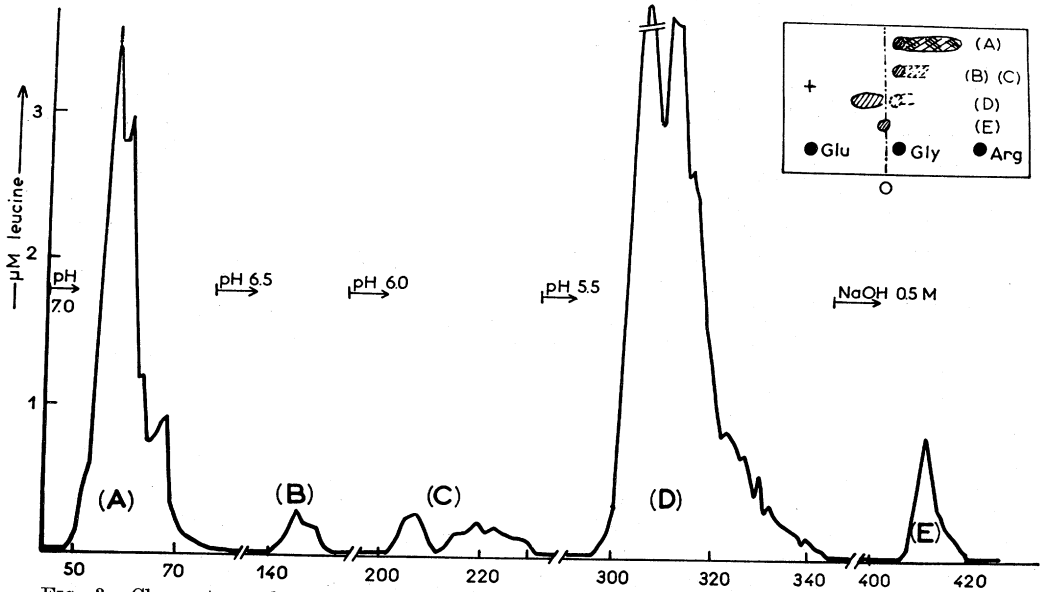

FIG. 3. Chromatography on DEAE-cellulose (100 by 1 cm) of the substances soluble at pH 4.6 obtained from heated cow κ-casein (120 C—20 min) 0.5 M pyridine–acetic acid buffers of varying pH. Upper right diagram: electrophoretic pattern of the different fractions (Whatman no. 1; 20 v/cm; pH 6.5; 2 hr.)

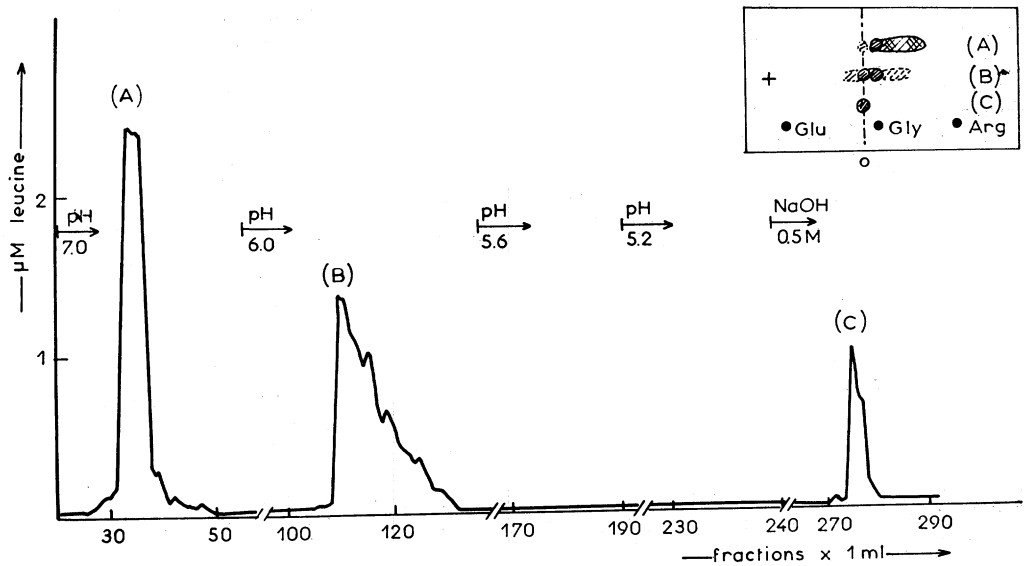

FIG. 4. Chromatography on DEAE-cellulose of substances soluble at pH 4.6 obtained from non-heated cow κ-casein (see legend of Fig. 3).

heated κ-casein, and there were large differences in the relative proportions of the fractions, as indicated in Table 3. Nearly all the peptidic

TABLE 3

Fractionation of the soluble substances obtained at pH 4.6 from native or heated κ-casein

	Heated 120 C—20 min	Control
Total amount of soluble substances *(% of κ-casein)*	5.8	1.5
Chromatographic fractions[a] *(% of the total eluted material)*		
At pH:		
7.0	25 (A)	41 (A)
6.5 and 6.0	5 (B,C)	50 (B)
5.5	67 (D)	0
0.5 N NaOH	3 (E)	9 (C)

[a] See Figs. 3 and 4.

substances from nonheated κ-casein were eluted at pH 7.0 and 6.0, and no ninhydrin positive substances were found at a lower pH. On the other hand, most of the peptidic substances from heated κ-casein were eluted at pH 5.5; furthermore, they were mainly negatively charged peptides. The second part of Peak D (Fig. 3) was separated, and it contained a substance which had the same electrophoretic behavior as the rennin caseino-glycopeptide. Its amino acid composition was determined, and the number of amino acid residues (calculated as before) is shown in Table 2. There is much similarity between this peptide and the rennin caseino-glycopeptide, and the heat caseino-glycopeptide b). The glucidic composition of these peptides was not determined, but they contained sugars, as they gave a positive reaction with the Schiff reagent after periodic oxidation.

Discussion

The high molecular weight of the peptides liberated by heat explains their difficult separation by gel filtration. These peptides seem to be strongly absorbed on intact or residual casein. This observation and the presence of sugars in some fractions may explain why molecular sieving was not effective. However, it was possible by filtration on Sephadex G-50 of heated whole casein to characterize a substance of low solubility, which was very similar to paracasein obtained after rennin digestion of casein (2).

We have studied mainly the heat degradation of κ-casein, as this casein component has a characteristic composition and plays a major role in the clotting processes. By three different ways (TCA-precipitation, preparative electrophoresis, and chromatography on an anion-exchanger) we isolated a peptide having the main characteristic properties of the rennin caseino-glycopeptide. Thus, the action of rennin and that of heat (120 C for 20 min) seem to be similar.

These experiments allow us to suggest that the peptidic chain of κ-casein contains a particularly labile linkage, easily split not only by rennin but also by other, even nonenzymatic treatments (9–11).

The hydrolytic action of heat has not, of course, the specificity of rennin. The three main casein fractions (α_s, β, κ) were attacked during heating, but the process was probably different in each case. β-Casein was the most resistant to heat action, as was concluded from the proportion of nonprotein substances and from the electrophoretic patterns. The degradation of α_s-casein was more intensive and more degradation products were observed (11).

Acknowledgments

This research was supported in part by Grant FG-Fr-112 from the United States Department of Agriculture.

The technical assistance of Misses B. Brégeard and N. Berthelot is gratefully acknowledged.

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